View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Elsevier - Publisher Connector

FEBS Letters 580 (2006) 1833–1838

Reduced activity of the hypertension-associated Lys528Arg mutant of human adipocyte-derived leucine aminopeptidase (A-LAP)/ER-aminopeptidase-1

Yoshikuni Goto[a,b], Akira Hattori[a], Yasuhiro Ishii[b], Masafumi Tsujimoto[a,*]

[a] *Laboratory of Cellular Biochemistry, RIKEN, 2-1 Hirosawa, Wako-shi, Saitama 351-0198, Japan*
[b] *Laboratory of Applied Protein Chemistry, Tokyo University of Agriculture and Technology, Fuchu, Tokyo 183-0054, Japan*

Received 15 December 2005; revised 21 January 2006; accepted 15 February 2006

Available online 24 February 2006

Edited by Irmgard Sinning

Abstract **The adipocyte-derived leucine aminopeptidase (A-LAP)/ER aminopeptidase-1 is a multi-functional enzyme belonging to the M1 family of aminopeptidases. It was reported that the polymorphism Lys528Arg in the human *A-LAP* gene is associated with essential hypertension. In this study, the role of Lys528 in the enzymatic activity of human A-LAP was examined by site-directed mutagenesis. Among non-synonymous polymorphisms tested, only Lys528Arg reduced enzymatic activity. The replacement of Lys528 with various amino acids including Ala, Met, His and Arg caused a significant decrease in the enzymatic activity. Molecular modeling of the enzyme suggested that Lys528 is located near the entrance of the substrate pocket. These results suggest that Lys528 is important for maximal activity of A-LAP by maintaining the appropriate structure of the substrate pocket of the enzyme. The reduced enzymatic activity of A-LAP may cause high blood pressure and the observed association between the polymorphism and hypertension.**
© 2006 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

Keywords: Aminopeptidase; Angiotensin II; Kallidin; Polymorphism; Site-directed mutagenesis

1. Introduction

Aminopeptidases hydrolyze the N-terminal amino acids of proteins or peptide substrates. They are distributed widely in animal and plant tissues as well as in bacteria and fungi, suggesting that they play important roles in various biological processes [1]. Previously, we cloned a cDNA for placental leucine aminopeptidase (P-LAP)/oxytocinase, a type II membrane-spanning protein with a relatively long N-terminal cytosolic domain [2]. Subsequently, we cloned cDNAs encoding adipocyte-derived leucine aminopeptidase (A-LAP) and leukocyte-derived arginine aminopeptidase (L-RAP) by searching databases [3,4]. Structural and phylogenetic analyses indicated that P-LAP, A-LAP and L-RAP are most closely related among the M1 family of aminopeptidases, which contain GAMEN and $HEXXH(X)_{18}E$ consensus motifs. Therefore we proposed that they should be classified into "the oxytocinase subfamily of M1 aminopeptidases" [5].

A-LAP is a multi-functional aminopeptidase shown to play roles in the regulation of blood pressure, angiogenesis and antigen presentation to MHC class I molecules [5–9]. Moreover, this enzyme was shown to bind to cytokine receptors such as TNF type I receptor, interleukin (IL)-6 α-receptor, and IL-1 type II receptor, and promote ectodomain shedding of these receptors [10–12]. A-LAP is also designated puromycin-insensitive leucine-specific aminopeptidase (PILS-AP) [13], ER-aminopeptidase associated with antigen presentation (ERAAP) [8], ER-aminopeptidase (ERAP)-1 [9] and aminopeptidase regulator of TNFR1 shedding (ARTS-1) [10] due to its multi-functional properties.

Since A-LAP cleaves and inactivates angiotensin II and converts kallidin to bradykinin efficiently, it was suggested that the enzyme plays a role in the regulation of blood pressure [14]. By screening for polymorphisms in the human *A-LAP* gene, Yamamoto et al. identified the association of Lys528Arg with essential hypertension and hypothesized that mutant A-LAP with Lys528Arg is less active than the wild-type enzyme [6]. It was also reported that this polymorphism determines the degree of regression of left ventricular hypertrophy during antihypertensive treatment in patients with essential hypertension and left ventricular hypertrophy [15]. These results suggest that Lys528 of wild-type A-LAP plays a role in the enzymatic action of the enzyme.

In the current study, we have examined the importance of this residue in the enzymatic action of A-LAP by mutational analysis. Our data indicate that Lys528 is indeed important for the maximal activity of A-LAP by maintaining high affinity-binding of the substrate to the enzyme.

2. Materials and methods

2.1. Site-directed mutagenesis

The cDNAs encoding for mutant A-LAPs were generated by two-step PCR. PCRs were carried out in 0.2 ml tubes with a 30-μl reaction volume. The sequences of sense oligonucleotide primers used to introduce point mutations in A-LAP cDNA, are listed in Table 1. First PCRs were carried out for 1 cycle at 98 °C for 3.5 min, followed by 10 cycles at 95 °C for 0.5 min, at 55 °C for 1 min, and at 72 °C for 2 min using *Pyrobest*

*Corresponding author. Fax: +81 48 462 4670.
E-mail address: tsujimot@riken.jp (M. Tsujimoto).

Abbreviations: A-LAP, adipocyte-derived leucine aminopeptidase; LTA4H, leukotriene A_4 hydrolase; NA, β-naphtylamide; L-RAP, leukocyte-derived arginine aminopeptidase; P-LAP, placental leucine aminopeptidase; TIFF3, Tricorn-interacting factor F3

0014-5793/$32.00 © 2006 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.
doi:10.1016/j.febslet.2006.02.041

Table 1
Sequences of PCR primer used for site-directed mutagenesis

Mutation	Primer sequence[a]
Glu56Lys	CGACTTCCTAAGTACGTCATCCCAGTTC
Pro127Arg	CTGGAACACCCCCGTCAGGAGCAAATTG
Ile276Met	CAGACAAGATGAATCAAGCAGATTATG
Lys528Ala	CTTGGACACTGCAGGCAGGTTTTCCCCTAAC
Lys528Met	CTTGGACACTGCAGATGGGTTTTCCCCTAAC
Lys528His	CTTGGACACTGCAGCATGGTTTTCCCCTAAC
Lys528Arg	CTTGGACACTGCAGAGAGGTTTTCCCCTAAC
Asp575Asn	CACCAGCAAATCCAACATGGTCCATCGAT
Gln730Glu	GAATGCTGCGGAGTGAACTACTACTCCTC

The underlined bases encode a new amino acid.
[a]Primer sequences for the sense strand are shown.

DNA polymerase (Takara, Kyoto Japan). Sense primer A (5′-CACCAACCCTAAAAAACCGCCACCATGGTGTTTCTGCCC-3′) containing a CACC sequence for directional cloning and initiation ATG codon and antisense primers complimentary to the sequences listed in Table 1 were employed for the amplification of upper stream fragments. Down stream fragments with the sequence for 6 × His tag at the 3′-end were amplified using mutagenic sense primers and primer B (5′-GACTGTCGACTTAGTGATGGTGATGGTGATGCATACGTTCAAGCTT-3′).

The two products of first PCR were used as templates for the second PCR. Second PCRs were carried out with primers A and B for 1 cycle at 98 °C for 3.5 min, followed by 25 cycles at 95 °C for 0.5 min, at 55 °C for 1 min, and at 72 °C for 2 min. The resultant products were inserted into the entry vector, pENTR-D-TOPO, using a TOPO-cloning system (Invitrogen). The sequences of the products were confirmed by automated sequencing on an Applied Biosystems model 377.

2.2. Expression and purification of recombinant wild-type and mutant A-LAPs in the baculovirus system

Human wild-type and mutant A-LAPs with C-terminal His tags inserted into pENTR-D-TOPO were transfected to the destination vector pDEST8 via a LR reaction (Invitrogen). The pDEST8 vectors containing A-LAP cDNAs were transformed to competent DH10bac *Escherichia coli* cells harboring the baculovirus genome (bacmid) and a transposition helper vector (Invitrogen). Subsequently, insect Sf9 cells were transfected with recombinant bacmids using the Cellfectin reagent (Invitrogen). After a 3-day incubation period, recombinant baculoviruses were isolated and used to infect Sf9 cells at a multiplicity of infection of 0.1. Three days after infection, the amplified viruses were harvested.

For the production of A-LAPs, Sf9 cells were grown at 27 °C in 100 ml of Sf-900II medium (Gibco) and 1.5×10^6 cells/ml were infected at a multiplicity of infection of 1–3. After 3 days, the culture medium was collected after centrifugation at $5000 \times g$ for 15 min.

The culture medium was loaded onto a hydroxyapatite (Toyobo, Osaka, Japan) column (bed volume: 10 ml) pre-equilibrated with 5 mM phosphate buffer (pH 7.5). After extensive washing with the same buffer, A-LAP was eluted from the column with 100 mM phosphate buffer (pH 7.5). The eluate was then loaded onto a Co^{2+}-chelating Sepharose (Pharmacia) column (bed volume: 1 ml) pre-equilibrated with 10 mM phosphate buffer (pH 7.5) containing 0.1 M NaCl. After extensive washing with 10 mM phosphate buffer (pH 7.5) containing 0.1 M NaCl and 5 mM imidazole, A-LAP was eluted with 10 mM phosphate buffer (pH 7.5) containing 0.1 M NaCl and 100 mM imidazole. The A-LAP-containing fractions were extensively dialyzed against 25 mM Tris–HCl buffer (pH 7.5) containing 0.125 M NaCl and concentrated with a ultrafiltration membrane and stored at −20 °C prior to use.

2.3. Measurement of aminopeptidase activity

A-LAP activity was determined with a synthetic fluorogenic substrate, L-leucyl-β-naphtylamide (Leu-NA) (Bachem, Bubendorf, Switzerland). The reaction mixture containing various concentrations of Leu-NA and either wild-type or mutant A-LAPs (100 ng/ml) in 500 μl of 20 mM phosphate buffer (pH 7.5) containing 10 μg/ml bovine serum albumin (BSA) was incubated at 37 °C for 5 min. After the incubation, the amount of β-naphtylamine released was measured by spectrofluorophotometry (F-2000, Hitachi) at an excitation wavelength of 335 nm and an emission wavelength at 410 nm. The kinetic parameters (K_m and V_{max}) were calculated from Lineweaver–Burk plots. The results were represented by K_m, relative k_{cat} and relative k_{cat}/K_m values. All measurements were performed in triplicate.

2.4. Cleavage of peptide hormones by recombinant A-LAPs

Peptide hormones (Peptide Institute, Osaka, Japan) (25 μM) were incubated with either wild-type or mutant A-LAPs (2 μg/ml) at 37 °C in 20 mM phosphate buffer (pH 7.5) containing 10 μg/ml BSA. The reaction was terminated by addition of 2.5% (v/v) formic acid. The peptides generated were separated by reverse-phase HPLC on a COSMOSIL (4.6 × 250 mm) column (Nacalai Tesque, Kyoto, Japan) using a Gilson HPLC system with a Hewlett-Packard HP 1040A diode-array detector. Peptides generated from angiotensin II or kallidin were eluted isocratically with 20% acetonitrile in 0.09% trifluoroacetic acid at a flow rate of 0.5 ml/min. The molecular masses of peptides were determined by matrix-assisted laser desorption ionization time of flight (MALDI-TOF) MS with a REFLEX mass spectrometer (Bruker-Franzen Analytik) using 2-mercaptobenzothiazole as the matrix.

2.5. Molecular modeling of human A-LAP

The recently published X-ray crystallographic structures of human leukotriene A_4 hydrolase (LTA4H) [16] and *Thermoplasma acidophilum* Tricorn-interacting factor F3 (TIFF3) [17] were used as templates for modeling the substrate pocket of human A-LAP using SWISS-MODEL Internet server (http://www.expasy.org/swissmod/). The structure was displayed using the CueMol program (Ishitani, R., CueMol: Molecular Visualization Framwork, http://cuemol.sourceforge.jp).

2.6. Materials

Aminopeptidase inhibitors (amastatin, bestatin and oxidized leucinethiol) were purchased from Sigma. Oxidized leucinethiol (Leu-SH) was reduced by dithiothreitol before use.

3. Results and discussion

Until now, there are 33 polymorphisms in the human *A-LAP* gene and 10 non-synonymous polymorphisms are reported [6]. Among them, A1533G caused the substitution of Lys528 to arginine. (Hereafter we refer to the mutant resulting from this polymorphism as Lys528Arg A-LAP.) It has been reported that Lys528Arg A-LAP is associated with essential hypertension [6]. We have shown that A-LAP can inactivate angiotensin II and convert kallidin (Lys-bradykinin) to bradykinin in vitro [14]. These results prompted us to examine the enzymatic properties of the wild-type and mutant enzymes derived from non-synonymous polymorphisms reported in Ref. [6]. Our initial experiments showed that transcriptional and translational rates of the non-synonymous polymorphic genes are rather constant (data not shown). Therefore it is reasonable to speculate that reduced A-LAP activity may cause hypertension.

To examine the hypothesis, wild-type and mutant A-LAPs were transiently expressed in a baculovirus system and purified to homogeneity on SDS–PAGE (Fig. 1A). All the enzymes showed single bands with apparent molecular weights of ~110 kDa as expected. We used these preparations to measure the enzymatic activity of the enzymes thereafter.

Table 2 shows the enzymatic activities of wild-type and mutant A-LAPs using Leu-NA as a substrate. A significant reduction of enzymatic activity (shown by relative k_{cat}/K_m) was seen only in Lys528Arg A-LAP. The other mutant enzymes largely retained the activity toward this synthetic substrate. Nearly the

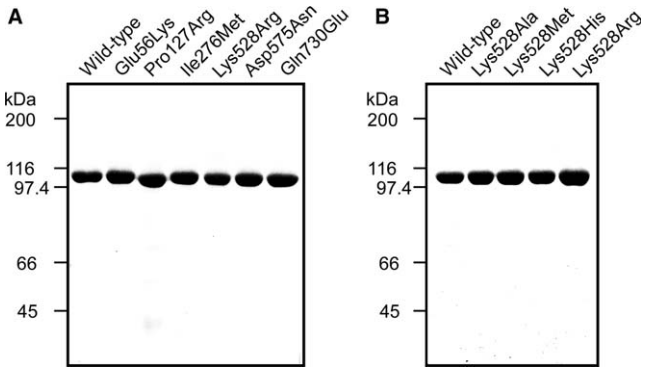

Fig. 1. SDS–PAGE of purified recombinant wild-type and mutant A-LAPs.

Table 2
Kinetic parameters of wild-type and mutant A-LAPs

Mutant	SNP	K_m for Leu-NA[a] (μM)	k_{cat}[a] (%)	k_{cat}/K_m[a] (%)
Wild-type	Major allele	685 ± 38	100	100
Glu56Lys	G166A	595 ± 98	113 ± 8	130 ± 13
Pro127Arg	C380G	730 ± 170	131 ± 11	123 ± 13
Ile276Met	A828G	461 ± 48	84 ± 7	120 ± 10
Lys528Arg	A1583G	2040 ± 201	74 ± 8	25 ± 1
Asp575Asn	G1723A	959 ± 92	170 ± 6	121 ± 5
Gln730Glu	C2188G	723 ± 37	118 ± 5	112 ± 2
Lys528Ala	–	2998 ± 402	97 ± 9	22 ± 1
Lys528Met	–	2936 ± 115	101 ± 6	23 ± 1
Lys528His	–	2976 ± 474	65 ± 6	15 ± 4

[a]The values are means ± S.D. (n = 3).

same result was obtained when another synthetic substrate, Met-NA, was employed (data not shown). These results indicate that among non-synonymous polymorphisms observed in the *A-LAP* gene, only Lys528Arg polymorphism causes a significant decrease in the enzymatic activity of the resultant mutant A-LAP.

Next we compared the hydrolytic activities of various A-LAPs toward natural hormones (Fig. 2). We chose angiotensin II and kallidin as substrates in this study because among peptide hormones that can be substrates of A-LAP in vitro, these two hormones are involved in the regulation of blood pressure [14]. Fig. 2A shows the cleavage of angiotensin II by the wild-type and Lys528Arg A-LAPs. Analysis of the degradation products showed that the wild-type enzyme cleaved the N-terminal Asp residue efficiently in our assay system and the generation of angiotensin III was clearly detected within 1 h. The appearance of angiotensin IV was also detected through subsequent cleavage of the Arg residue. On the other hand, Lys528Arg A-LAP was less active toward the hormone and a ~60% decrease in the generation of angiotensin III was observed. No angiotensin IV was detected during the incubation period. When the conversion of kallidin to bradykinin was measured, it was found that Lys528Arg A-LAP was also less active than the wild-type enzyme and a ~70% decrease in the conversion was observed (Fig. 2B). These results suggest that Lys528Arg A-LAP is less active than wild-type A-LAP toward natural hormones that can be substrates of the enzyme.

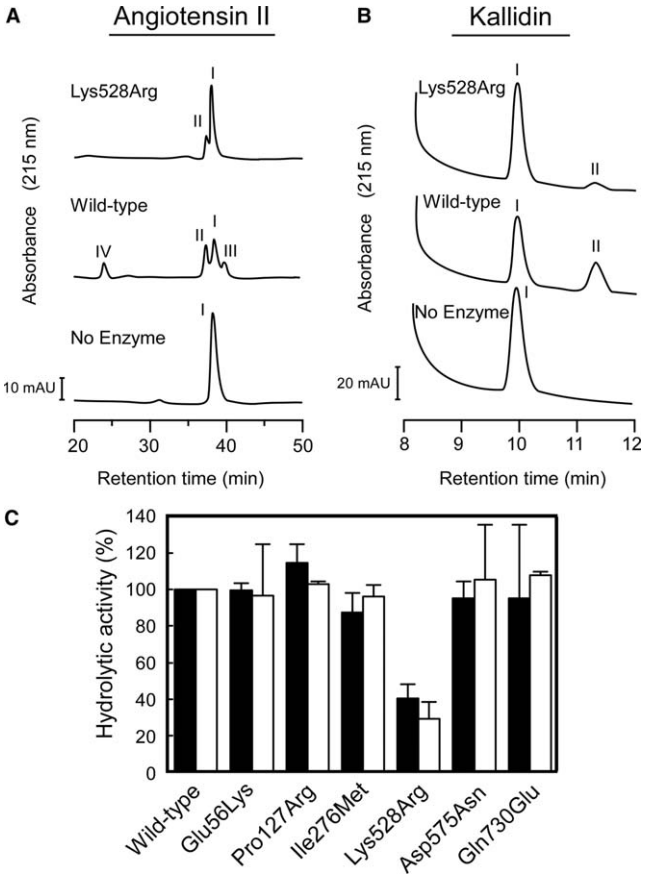

Fig. 2. Enzymatic activity of wild-type and mutant A-LAPs toward peptide hormones. (A) Cleavage of angiotensin II. Angiotensin II (25 μM) was incubated with either wild-type or Lys528Arg mutant A-LAP (2 μg/ml) at 37 °C for 60 min. The peptides generated were loaded onto an HPLC column and separated as described in Section 2. (I: angiotensin II; II: angiotensin III; III: angiotensin IV; IV: de-[Val]-angiotensin IV.) (B) Conversion of kallidin to bradykinin. Kallidin (25 μM) was incubated with either wild-type or Lys528Arg mutant A-LAP (2 μg/ml) at 37 °C for 20 min. The peptides generated were loaded onto an HPLC column and separated as described in Section 2. (I: kallidin; II: bradykinin.) (C) Cleavage of angiotensin II and kallidin by wild-type and mutant A-LAPs. 25 μM of angiotensin II (closed bar) and kallidin (open bar) were incubated with various A-LAPs (2 μg/ml) at 37 °C for 60 min and 20 min, respectively. The activity of wild-type A-LAP was taken as 100%.

Fig. 2C summarizes the hydrolytic activities of the wild-type and various mutant A-LAPs toward angiotensin II and kallidin. As in the case of synthetic substrates, only Lys528Arg A-LAP showed decreased activity toward the hormones. Other mutants tested showed little difference from the wild-type enzyme. These results suggest that the association of this polymorphism in the *A-LAP* gene with hypertension was at least in part due to the reduced enzymatic activity of the mutant enzyme.

To elucidate the significance of Lys528 in the enzymatic activity of A-LAP, this residue was changed to various amino acids. All mutants were transiently expressed in a baculovirus system and purified to homogeneity (Fig. 1B).

As shown in Table 2, all mutants showed decreased activities toward Leu-NA when compared with the wild-type enzyme. These results suggest that Lys528 is required for the maximal activity of the enzyme. It is notable that all mutants showed

2–3-fold increase in K_m values, indicating that this residue participates in the determination of substrate-binding affinity to the enzyme.

We then compared the hydrolytic activities of wild-type and various Lys528-replaced mutant A-LAPs toward angiotensin II and kallidin (Fig. 3). As shown in the figure, all mutants were less active than the wild-type enzyme. Of note, a naturally occurring Lys528Arg A-LAP was less active than the other mutants tested. These results support the notion that Lys528 is necessary for maximal activity of the enzyme by maintaining the affinity for substrates.

We next tried to estimate the role of this residue in the enzymatic action by measuring the K_i values of various aminopeptidase inhibitors for the wild-type and Lys528Arg A-LAPs. As shown in Table 3, amastatin and bestatin showed a 2.90- and 5.38-fold increase in the K_i value for Lys528Arg A-LAP, respectively. In contrast, no difference was observed when Leu-SH was used as an inhibitor. We also examined the effects of inhibitors on the activities of Glu320Gln and His357Phe A-LAPs. While Glu320 in the GAMEN motif is important for the interaction with N-terminal α-amino group of peptide substrate, His357 in HEXXH motif is a Zn^{2+}-binding ligand [18,19]. The inhibitory activities of amastatin and bestatin were much less to both mutants than those to wild-type and Lys528Arg A-LAPs. Moreover, significant increases in the K_i values of Leu-SH for both mutants (22.5- and 169-fold increase to Glu320 and His357, respectively) were observed. These results suggest the different role of Lys528 from Glu320 and His357, both of which are important for the integrity of substrate pocket structure.

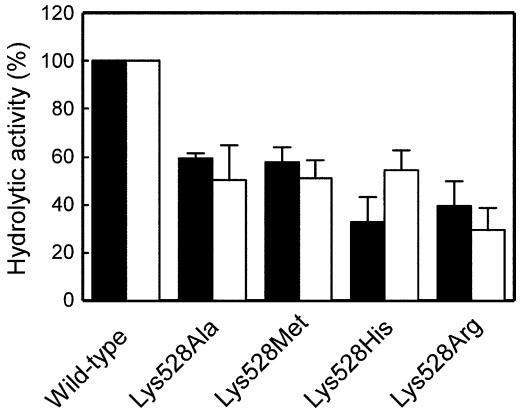

Fig. 3. Cleavage of angiotensin II and kallidin by the wild-type and Lys528 mutant A-LAPs. 25 μM of angiotensin II (closed bar) and kallidin (open bar) were incubated with various A-LAPs (2 μg/ml) at 37 °C for 60 min and 20 min, respectively. The activity of wild-type A-LAP was taken as 100%.

Alignment of the M1 family members indicates that Lys528 is conserved only in the oxytocinase subfamily (Fig. 4A). Therefore it is possible that the role of this residue in the enzymatic activity is common to the subfamily members. Recently, the crystal structures for LTA4H and TIFF3 were solved [16,17]. Human LTA4H and *T. acidophilum* TIFF3 show 11.8% and 22.2% similarity to human A-LAP, respectively. The sequence similarity of these two proteins to A-LAP allowed us to model the structure of A-LAP using these two enzymes as templates (Fig. 4B). Modeling of the substrate pocket of A-LAP reveals that Lys528 is clustered with other amino acid residues required for maximal activity (i.e., His417 [20] and His548 (our unpublished result)) near the entrance of the pocket. We speculate that interactions between these residues contribute to the high affinity-binding of substrate to the enzyme by maintaining the appropriate shape of the substrate pocket of the enzyme. Taken together, it is quite conceivable that Lys528 is important for the formation and maintenance of the entrance of substrate pocket of the enzyme. For a detailed analysis of the mode of enzymatic action of A-LAP, we have to await the determination of the three-dimensional structure of the enzyme by X-ray crystallography.

Since only the crystal structure of LTA4H and TIFF3 have been resolved among the M1 family of aminopeptidases, essential amino acid residues for the enzymatic activity of the family members were identified by molecular modeling and site-directed mutagenesis [18–26]. These studies usually identified conserved residues among M1 family members. In this study, we have identified Lys528 as a residue affecting the enzymatic activity of A-LAP that was not identified in the previous studies. The significance of this residue was initially suggested by an analysis reporting the association of the Lys528Arg polymorphism in the *A-LAP* gene with essential hypertension [6]. Our data provide the molecular bases of the association between polymorphisms of the *A-LAP* gene and essential hypertension in that Lys528Arg A-LAP is less active than the wild-type enzyme due to a reduction in affinity for substrate hormones. It is plausible that the reduced cleavage of substrate peptides such as angiotensin II and kallidin may result in high blood pressure and the observed association between the polymorphism and hypertension. To further analyze the pathophysiological significance of the polymorphism, it would also be interesting to compare the antigen presentation activities of wild-type and mutant A-LAPs to MHC class I molecules [8,9].

Table 3
K_i values of aminopeptidase inhibitors for wild-type and Lys528Arg A-LAPs

	Amastatin		Bestatin		Leucinethiol	
	K_i[a]		K_i[a]		K_i[a]	
	(μM)	(fold)	(μM)	(fold)	(μM)	(fold)
Wild-type	20.0 ± 5.0	(1.00)	53.7 ± 0.8	(1.00)	0.15 ± 0.02	(1.00)
Lys528Arg	57.9 ± 8.4	(2.90)	289 ± 40	(5.38)	0.16 ± 0.01	(1.07)
Glu320Gln	205 ± 12	(10.3)	526 ± 3	(9.80)	3.38 ± 0.43	(22.5)
His357Phe	162 ± 20	(8.10)	507 ± 26	(9.44)	25.4 ± 5.2	(169)

[a]The values are means ± S.D. (n = 3).

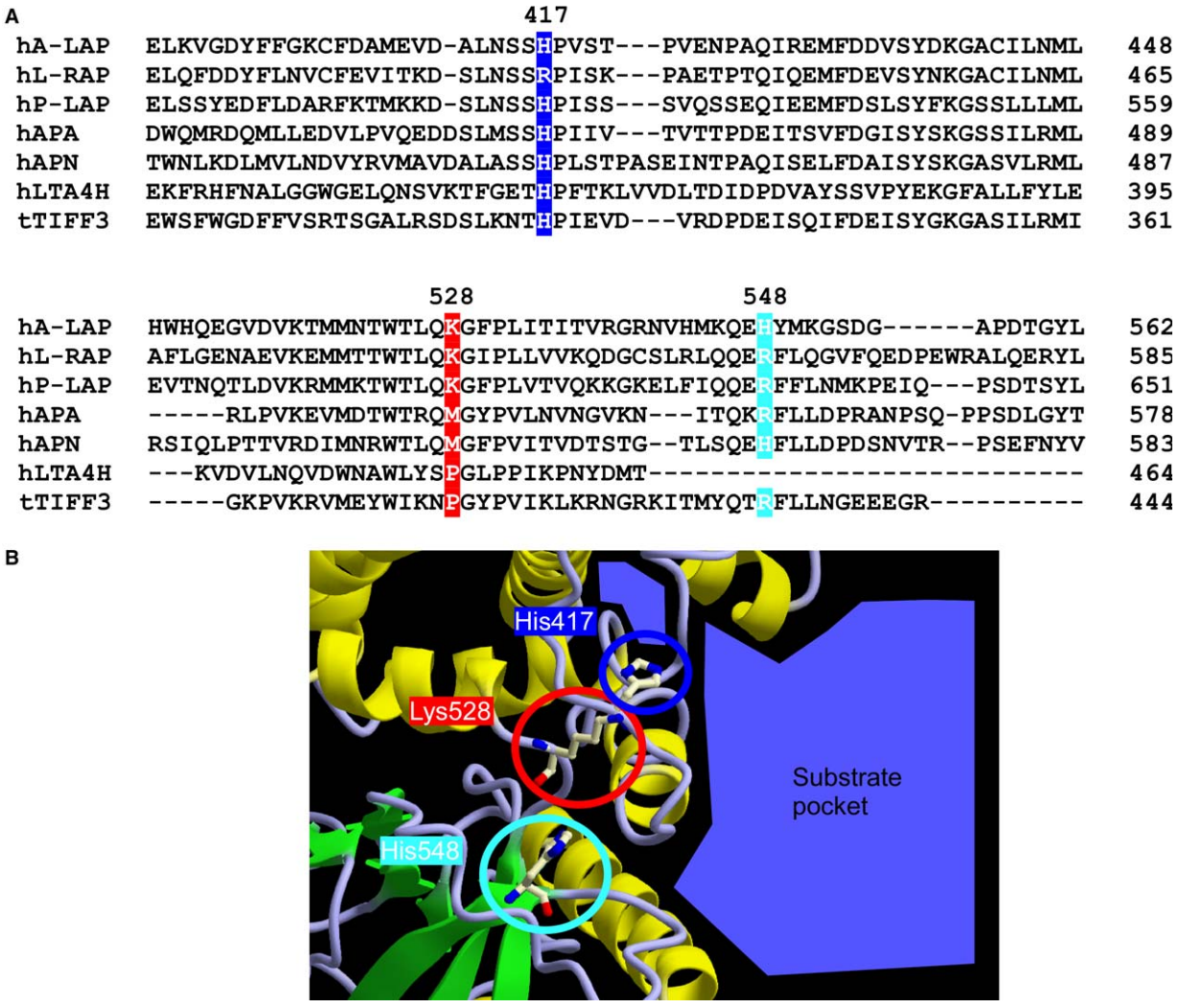

Fig. 4. Molecular modeling of human A-LAP. (A) Alignment of the human A-LAP amino acid sequence with the sequences of other human aminopeptidases belonging to the M1 family and *T. acidophilum* TIFF3. APA: aminopeptidase A, APN: aminopeptidase N. (B) Modeling of the substrate pocket of human A-LAP using human LTA4H and *T. acidophilum* TIFF3 as templates.

Acknowledgements: This work was supported in part by grants-in-aid from the Ministry of Education, Science, Sports and Culture of Japan and grants for the "Chemical Biology Research Program" from RIKEN and from The Naito Foundation.

References

[1] Taylor, A. (1993) Aminopeptidases: structure and function. FASEB J. 7, 290–298.
[2] Rogi, T., Tsujimoto, M., Nakazato, H., Mizutani, S. and Tomoda, Y. (1996) Human placental leucine aminopeptidase: a new member of type II membrane-spanning zinc metallopeptidase family. J. Biol. Chem. 271, 56–61.
[3] Hattori, A., Matsumoto, H., Mizutani, S. and Tsujimoto, M. (1999) Molecular cloning of adipocyte-derived leucine aminopeptidase highly related to placental leucine aminopeptidase/oxytocinase. J. Biochem. (Tokyo) 125, 931–938.
[4] Tanioka, T., Hattori, A., Masuda, S., Nomura, Y., Nakayama, H., Mizutani, S. and Tsujimoto, M. (2003) Human leukocyte-derived arginine aminopeptidase: the third member of the oxytocinase subfamily of aminopeptidases. J. Biol. Chem. 278, 32275–32283.
[5] Tsujimoto, M. and Hattori, A. (2005) The oxytocinase subfamily of M1 aminopeptidases. Biochim. Biophys. Acta 1751, 9–18.
[6] Yamamoto, N., Nakayama, J., Yamakawa-Kobayashi, K., Hamaguchi, H., Miyazaki, R. and Arinami, T. (2002) Identification of 33 polymorphisms in the adipocyte-derived leucine aminopeptidase (ALAP) gene and possible association with hypertension. Hum. Mutat. 19, 251–257.
[7] Miyashita, H., Yamazaki, T., Akada, T., Niizeki, O., Ogawa, M., Nishikawa, S. and Sato, Y. (2002) A mouse orthologue of puromycin-insensitive leucyl-specific aminopeptidase is expressed in endothelial cells and plays an important role in angiogenesis. Blood 99, 3241–3249.
[8] Serwold, T., Gonzalez, F., Kim, J., Jacob, R. and Shastri, N. (2002) ERAAP customizes peptides for MHC class I molecules in the endoplasmic reticulum. Nature 419, 480–483.
[9] Saric, T., Chang, S.-C., Hattori, A., York, I.A., Markant, S., Rock, K.L., Tsujimoto, M. and Goldberg, A.L. (2002) An IFN-γ-induced aminopeptidase in the ER, ERAPI, trims precursors to MHC class I-presented peptides. Nat. Immunol. 3, 1169–1176.
[10] Cui, X., Hawari, F., Alsaary, S., Lawrence, M., Combs, C.A., Geng, W., Rouhani, F.N., Miskinis, D. and Levine, S.J. (2002) Identification of ARTS-1 as a novel TNFR1-binding protein that promotes TNFR1 ectodomain shedding. J. Clin. Invest. 11, 515–526.
[11] Cui, X., Rouhani, F.N., Hawari, F. and Levine, S.J. (2003) ARTS-1 is required for interleukin-6 receptor shedding. J. Biol. Chem. 278, 28677–28685.

[12] Cui, X., Rouhani, F.N., Hawari, F. and Levine, S.J. (2003) Shedding of the type II IL-1 decoy receptors requires a multifunctional aminopeptidase, aminopeptidase regulator of TNF receptor type 1 shedding. J. Immunol. 171, 6814–6819.
[13] Schomburg, L., Kollmus, H., Friedrichsen, S. and Bauer, K. (2000) Molecular characterization of a puromycin-insensitive leucyl-specific aminopeptidase, PILS-AP. Eur. J. Biochem. 267, 3198–3207.
[14] Hattori, A., Kitatani, K., Matsumoto, H., Miyazawa, S., Rogi, T., Tsuruoka, N., Mizutani, S., Natori, S. and Tsujimoto, M. (2000) Characterization of recombinant human adipocyte-derived leucine aminopeptidase expressed in Chinese hamster ovary cells. J. Biochem. (Tokyo) 128, 755–762.
[15] Hallberg, P., Lind, L., Michaelsson, K., Kurland, L., Kahan, T., Malmqvist, K., Ohman, K.P., Nystrom, F., Liljedahl, U., Syvannen, A. and Melhus, H. (2003) Adipocyte-derived leucine aminopeptidase genotype and response to antihypertensive therapy, BMC Cardiovasc. Disord. http://www.biomedcentral.com/1471-2261/3/11.
[16] Thunnissen, M.M.G.M., Nordlund, P. and Haeggstrom, J.Z. (2001) Crystal structure of human leukotriene A_4 hydrolase, a bifunctional enzyme in inflammation. Nat. Struct. Biol. 8, 131–135.
[17] Kyrieleis, O.J.P., Goettig, P., Kiefersauer, R., Huber, R. and Brandstetter, H. (2005) Crystal structure of the tricorn interacting factor F3 from *Thermoplasma acidophilum*, a zinc aminopeptidase in three conformations. J. Mol. Biol. 349, 787–800.
[18] Vazeux, G., Iturrioz, X., Corvol, P. and Llorens-Cortes, C. (1998) A glutamate residue contributes to the exopeptidase specificity in aminopeptidase A. Biochem. J. 334, 407–413.
[19] Lausten, P.G., Vang, S. and Kristensen, T. (2001) Mutational analysis of the active site of human insulin-regulated aminopeptidase. Eur. J. Biochem. 268, 98–104.
[20] Iturrioz, X., Vazeux, G., Celerier, J., Corvol, P. and Llorens-Cortes, C. (2000) Histidine 450 plays a critical role in catalysis and, with Ca^{2+}, contributes to the substrate specificity of aminopeptidase A. Biochemistry 39, 3061–3068.
[21] Luciani, N., Marie-Claire, C., Ruffet, E., Beaumount, A., Roques, B.P. and Fournie-Zaluski, M.-C. (1998) Characterization of Glu^{350} as a critical residue involved in the N-terminal amine binding site of aminopeptidase N (EC3, 4,11.2): insights into its mechanism of action. Biochemistry 37, 686–697.
[22] Papadopoulos, T., Kelly, J.A. and Bauer, K. (2001) Mutational analysis of the thyrotropin-releasing hormone-degrading ectoenzyme similarities and differences with other members of the M1 family of aminopeptidase and thermolysin. Biochemistry 40, 9347–9355.
[23] Vazeux, G., Iturrioz, X., Corvol, P. and Llorens-Cortes, C. (1997) A tyrosine residue essential for catalytic activity in aminopetidase A. Biochem. J. 327, 883–889.
[24] Iturrioz, X., Rozenfeld, R., Michaud, A., Corvol, P. and Llorens-Cortes, C. (2001) Study of asparagine 353 in aminopeptidase A: characterization of a novel motif (GXMEN) implicated in exopeptidase specificity of monozinc aminopeptidases. Biochemistry 40, 14440–14448.
[25] Rozenfeld, R., Iturrioz, X., Maigret, B. and Llorens-Cortes, C. (2002) Contribution of molecular modeling and site-directed mutagenesis to the identification of two structural residues, Arg-220 and Asp-227, in aminopeptidase A. J. Biol. Chem. 277, 29242–29252.
[26] Rozenfeld, R., Iturrioz, X., Okada, M., Maigret, B. and Llorens-Cortes, C. (2003) Contribution of molecular modeling and site-directed mutagenesis to the identification of a new, glutamate 215, involved in the exopeptidase specificity of aminopeptidase A. Biochemistry 42, 14785–14793.